

# UNCLASSIFIED

AD NUMBER
ADB253101
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Aug 99. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St, Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 27 Sep 2002

THIS PAGE IS UNCLASSIFIED

AD\_\_\_\_\_

GRANT NUMBER DAMD17-97-1-7092

TITLE: Role of the Integrin-Linked Kinase, ILK, in Mammary Carcinogenesis

PRINCIPAL INVESTIGATOR: Gregory E. Hannigan, Ph.D.

CONTRACTING ORGANIZATION: Hospital for Sick Children  
Toronto, Ontario, Canada M5G 1X8

REPORT DATE: August 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Aug 99). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

## NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

### LIMITED RIGHTS LEGEND

Award Number: DAMD17-97-1-7092

Organization: Hospital for Sick Children

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

---

Gregory E. Hannigan, Ph.D.

---

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE August 1999		3. REPORT TYPE AND DATES COVERED Annual (25 Jul 98 - 24 Jul 99)
4. TITLE AND SUBTITLE  Role of the Integrin-Linked Kinase, ILK, in Mammary Carcinogenesis			5. FUNDING NUMBERS  DAMD17-97-1-7092	
6. AUTHOR(S)  Hannigan, Gregory E., Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)  Hospital for Sick Children Toronto, Ontario, M5G 1X8 Canada			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES  Report Contains Colored Photographs				
12a. DISTRIBUTION / AVAILABILITY STATEMENT  Distribution authorized to U.S. Government agencies only (proprietary information, Aug 99). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words)  ILK-1 is a protein serine/threonine kinase which regulates integrin and growth factor signalling in epithelial cells. Recent evidence suggests that protein kinase B and glycogen synthase kinase 3- $\beta$ are ILK-1 substrates. ILK-1 has been shown to phosphorylate PKB on Ser473 <i>in vitro</i> , and to stimulate this phosphorylation <i>in vivo</i> . ILK-1 is thus a strong candidate phospholipid-dependent kinase 2 (PDK2) molecule. We have cloned a serine/threonine phosphatase belonging to the PP2C family, which forms stable complexes with p59 <sup>ILK-1</sup> protein in epithelial cells. This association is demonstrated in yeast two-hybrid experiments, <i>in vitro</i> 'pull-down' assays, and by co-immunoprecipitation from epithelial cell lysates. It is likely that this PP2C isoform specifically regulates ILK catalytic activity in these complexes, thereby suppressing the pro-apoptotic activity of PKB by inhibiting the PDK2 activity of ILK-1. A second ILK-1 interacting protein has been identified, which may provide insights into regulation of the actin cytoskeleton by integrins and ILK-1. This protein is ca. 35 kDa, comprised primarily of 2 calponin homology domains. These domains have been implicated in actin binding by a number of proteins, including spectrin, fodrin and $\alpha$ -actinin. This association is also likely to reflect a direct interaction with p59 <sup>ILK-1</sup> , detectable in yeast two-hybrid, and <i>in vitro</i> pull-down experiments.				
14. SUBJECT TERMS Breast Cancer      Signal transduction, integrin, extracellular matrix, epithelial biology Protein kinase, protein phosphatase, protein interactions			15. NUMBER OF PAGES 23	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified		18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified		19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified
				20. LIMITATION OF ABSTRACT Limited

## FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

\_\_\_\_ Where copyrighted material is quoted, permission has been obtained to use such material.

\_\_\_\_ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

\_\_\_\_ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

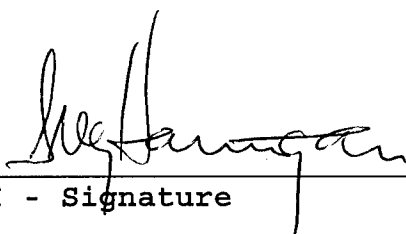
☒ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

\_\_\_\_ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

☒ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

☒ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

\_\_\_\_ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

  
\_\_\_\_  
PI - Signature

Spt 20/99  
\_\_\_\_  
Date

**TABLE OF CONTENTS**

Introduction	p. 2
Body	p. 2
Overview	p. 3
Results and Discussion	p. 3
A. Characterization of interacting clones	p. 3
1. 3B7, a putative actin-binding protein	p. 3
2. ILK-1 associated phosphatase: a novel PP2C	p. 5
a. Structure of ILKAP	p. 5
b. Expression of ILKAP in mammalian cells	p. 6
B. Development of retroviral vectors expressing ILK-1 variants	p. 7
Conclusions	p. 8
References	p. 9

**INTRODUCTION**

Recent data suggests ILK-1<sup>1</sup> regulates the Wnt signalling pathway by phosphorylating and inactivating glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ). As GSK3 $\beta$  activity regulates  $\beta$ -catenin levels and cellular localization, this interaction may at least in part explain the observed nuclear accumulation of  $\beta$ -catenin in ILK-1-overexpressing IEC-18 cells.<sup>2,3</sup> Fibronectin- and insulin-dependent activation of ILK-1 activity in epithelial cells has been shown to be blocked by inhibitors of phosphatidyl inositol-3' OH-kinase (PI-3-K), and constitutively activated by an activated catalytic p110 subunit of PI-3-K (Delcommen). PI-3-K dependent ILK-1 stimulation by these results in activation of Protein Kinase B (PKB/Akt) by phosphorylation on Ser473. Ser473 phosphorylation is effected by a phospholipid-dependent kinase 2 (PDK2) activity, which cooperates with phosphorylation of Thr308 by PDK1, to fully activate PKB. ILK-1 is thus a strong candidate PDK2 activity, and as such, may regulate PKB-dependent survival in cellular contexts other than loss of adhesion. Another recent study demonstrated cross-modulation between EGF receptor and  $\beta$ 1 integrins in human mammary epithelial HMT-3522 cells, the malignant T4 subclone was found to express activated p59<sup>ILK-1</sup>, relative to a non-malignant S1 subclone, although both expressed similar levels of p59<sup>ILK-1</sup>. Monoclonal antibodies inhibiting either  $\beta$ 1 integrin, or EGFR function were found to block the elevation of ILK-1 activity in T4 cells, without decreasing cellular p59<sup>ILK-1</sup> levels.<sup>4</sup> This results indicate that, in mammary epithelial cells, ILK-1 activation is critical to integrin-mediated signal transduction in response to external stimuli, or as a result of oncogenic progression.

**BODY****Overview**

The period covered by this report (August 1998 to August 1999) has been largely devoted to isolating and characterizing full length cDNA clones for the two ILK-1-interacting clones described in the previous report. Although this phase consumed a large amount of time and

effort, we are now in the position of having characterized full length cDNAs for the serine/threonine phosphatase (cDNA 5BT) and the putative actin-binding protein (cDNA 3B7). In the case of the phosphatase, stable cell lines have been established from human kidney epithelial HEK 293 cells which, either constitutively or conditionally, over-express this phosphatase. Co-immunoprecipitation experiments in 293 cells confirm that ILK-1 and the phosphatase associate in living cells, thus we have named the phosphatase ILKAP (ILK-1-associated phosphatase). Importantly, overexpression of ILKAP results in a G2/M block to cell cycle progression, and also blocks cell spreading on fibronectin and collagen matrices. ILKAP does not seem to affect cell adhesion on matrix proteins, and so the defect in cell spreading may relate to integrin-dependent changes in actin cytoskeleton rearrangements in response to ECM. Epithelial cells plated on fibronectin (FN) for one hour show a decrease in ILK-1 activity, as assayed on the exogenous substrate, myelin basic protein.<sup>1</sup> Recent evidence indicates that at earlier time points ILK-1 activity increases (15-20 min post FN), followed by a rapid down-modulation to sub-basal levels. These activation kinetics have been reproduced in our laboratory. The isolation of a putative serine-threonine phosphatase via interaction with ILK-1 has obvious implications for down-regulation of ILK-1 signalling. As persistent over-expression of ILK-1 activity in epithelial cells is tumourigenic, a negative regulator of ILK-1 activity would be a candidate tumour suppressor gene.

## Results and Discussion

### A. Characterization of ILK-1 interacting clones identified in the two-hybrid screen

#### 1. 3B7, encoding a putative actin-binding protein

We have screened yeast two-hybrid interaction libraries with bait proteins representing three variants of ILK-1 (see 1998 report). A total of  $1-2 \times 10^6$  transformants from each of two interaction libraries were independently screened with these baits. A HeLa epithelial cell library (obtained from Dr. Roger Brent, Massachusetts General Hospital) was screened with the baits LexILK-1, encoding a full length catalytically active ('wild-type') ILK-1 protein fused to the LexA DNA binding domain, and LexANK, encoding a truncated ILK-1 fusion protein which includes amino acid residues 1-276 of ILK-1. The full length, wild type ILK-1 bait trapped a 708bp partial cDNA, containing an open reading frame encoding 150 amino acid residues, and typical 3' end structure including an adenylation signal and polyA+ tract. The open reading frame suggested the protein encoded by this cDNA possessed a calponin homology domain (CH), which is a



common feature of actin-binding proteins, and which are thought to mediate actin-binding. However not all CH domain-containing proteins have been shown to bind actin, and it appears a minimum of two CH domains are required for actin binding function (Fig. 1).

We are currently proceeding with the characterization of the 3B7 transcripts and protein. Northern blots of polyA<sup>+</sup> RNA from a variety of human tissues (Clontech, MTN I blot), using 3B7 as a probe, indicate a complex pattern of transcripts of approximately 1.5, 1.7 and 6 kb, which are predominantly expressed in skeletal and cardiac muscle (See 1998 Report). By conceptually joining our 3B7 sequence with the overlapping sequences of two ESTs, we were able to design primer sets to investigate the linkage of these sequences in cellular mRNAs. RT-PCR reactions with the 5' EST and 3' 3B7 primers confirmed that the EST and 3B7 sequences are on the same transcript (not shown). A rabbit antiserum, raised against a 6xHis-tagged 3B7 fusion protein (HT-3B7), was affinity-purified over an immobilized HT-3B7 column. The resulting antibody preparations were tested for specificity in western blots against purified GST-3B7 and GST-ILK-1 fusion proteins. Our affinity-purified antibodies recognized the ca. 53 kDa GST-3B7 fusion protein (the GST moiety is 25 kDa), but not the GST-ILK-1 recombinant protein (not shown). The affinity-purified anti-3B7 recognized a single band from cytoplasmic lysates of human embryonic kidney HEK 293 cells, migrating at approximately 35 kDa in 10% SDS-PAGE (Fig. 2A). Adsorption of the antibody against GST-3B7 eliminated reactivity against the endogenous 35kDa band in HEK 293 lysates (Fig. 2B). The single band of 35 kDa suggests that the smallest of the 3B7-related transcripts seen in northern blot analyses encodes the expressed 3B7 protein. We have tentatively named this protein CLINT, for Cytoskeletal Linker to INTEGRINS. The significance of the larger transcripts is not known. They may encode proteins related to CLINT, which lack the 150 amino acids comprising the deduced C-terminal represented by the 3B7-encoded peptide. This may indicate alternative splicing of primary transcripts to produce CLINT-related proteins which we cannot detect with our C-terminal antibody.

GST-ILK recombinant fusion proteins were used in 'pull-down' assays to test the association of ILK and 3B7. CDNAs encoding full length wild-type and, kinase-inactive versions of ILK (E359K) were fused to GST on the pGEX4T-1 vector backbone (Pharmacia). Fusion proteins were incubated with lysates of *E.coli* BL21(DE3) transformed with the pProExHT-3B7 plasmid. Treatment of the BL21 cultures with 1mM IPTG results in induction of the HT-3B7 fusion protein to high levels, by derepression of the lacIq repressor. Both versions of ILK precipitated a small amount of HT-3B7 from uninduced lysates, presumably due to low level 'leakage' of the lacIq repressor. The association of HT-3B7 and GST-ILK proteins was greatly enhanced in the lysates from

IPTG-induced cultures (Fig. 3). These data indicate that ILK and the 3B7-encoded peptide form stable complexes *in vitro* and, in conjunction with the two-hybrid data, indicate that this association is likely to be direct.

## 2. ILKAP (ILK-1-associated phosphatase), a putative protein serine/threonine phosphatase of the PP2C family

### a) Structure of ILKAP

The LexANK-baited (ILK-1 residues 1-276) screen of the HeLa interaction library resulted in the isolation of a second interacting clone, which we have designated 5BT. When the 611 bp 5BT sequence was used to search the GenBank and EMBL databases (BLAST) highly significant matches to a number of protein serine/threonine phosphatases of the PP2C family were identified (Fig. 4). No other functional homologies were noted.

We have characterized a full length cDNA represented by the original 5BT isolate. The sequence of this clone indicates a functional protein serine/threonine phosphatase belonging to the PP2C family. A 1751 bp cDNA, H4C11, was cloned from an Origene rapid screen placental library. An open reading frame contained within this cDNA lined up with 95% amino acid identity with the published rat PP2C $\delta$  sequence. The 5' end structure of this cDNA was confirmed in the sequence of independently-cloned RT-PCR products, using primers derived from the 5' most region of the cDNA.

We have named the protein encoded by H4C11, ILKAP, for ILK-1-associated phosphatase. The structure of ILKAP is shown schematically in Fig 4. ILKAP comprises 392 amino acids, containing two PP2C boxes, 1 and 2. The N-terminal residues 115-206 comprise Box 1, which contains a DGH triplet which has been shown to be essential for catalytic activity of PP2C enzymes. Residues 215-392 comprise Box 2, which contains conserved residues among PP2C family members, and may be involved in substrate binding.<sup>5</sup> The region containing Box 2 is present in clones identified in the ILK-1-baited two-hybrid screen. Residues 1-114 of ILKAP do not score on homology searches against a number of protein databases (SWISSPROT, GENPEPT). A rat PP2C $\delta$  has been reported, which possesses 95% amino acid identity with ILKAP.<sup>6</sup> The boundaries of PP2C Boxes 1 and 2 are identical in ILKAP and rat PP2C $\delta$  and fifteen of the twenty residue differences between the rat PP2C $\delta$  and ILKAP occur between residues 1-114, suggesting strong evolutionary pressure to maintain the structure of ILKAP catalytic and substrate-binding regions.

### b) Expression of ILKAP and recombinant ILKAP in mammalian cells and tissues.

In order to survey a large number of human tissues for ILKAP, and ILK-1 expression, we probed a human multi-tissue expression array (MTE, Clontech) with ILK-1 and ILKAP cDNAs. Nylon membranes spotted with normalized amounts of RNA from 50 samples of pooled human tissues was probed with  $^{32}\text{P}$ -labelled ILKAP and ILK-1 cDNA probes. All 50 tissues expressed both ILK-1 and ILKAP (Fig. 5), as would be expected in light of the ubiquitous expression of  $\beta 1$  integrins. ILK-1 RNA levels appear highest in heart, aorta, colon, bladder, uterus, prostate, placenta and stomach, as well as exhibiting relatively strong expression levels in most fetal tissues sampled. ILKAP expression appears highest in the pancreas, pituitary and adrenal glands, stomach, small intestine, placenta, and fetal liver. The apparent lack of quantitative correlation between ILK-1 and ILKAP mRNA levels may not reflect protein levels in these tissues, and we do not know the stoichiometric ratio of  $\text{p}59^{\text{ILK-1}}$  in association with  $\text{p}47^{\text{ILKAP}}$  (see below) at this time. Thus the important result is that ILK-1 and ILKAP mRNAs are co-expressed in a wide variety of tissues.

IN SDS-PAGE, our affinity purified anti-ILKAP polyclonal antibodies recognize a single band, migrating with an apparent molecular weight of 47 kDa (Fig. 6). We tested the ability of ILKAP to associate with  $\text{p}59^{\text{ILK-1}}$  in mammalian cells by performing pull-down assays with the 6xHis-tagged ILKAP recombinant protein, HT-17Ai. The recombinant HT-17Ai protein efficiently complexed with endogenous  $\text{p}59^{\text{ILK-1}}$  in mouse C2C12 fibroblast lysates (Fig. 6A). Anti-ILK-1 antibodies precipitated  $\text{p}47^{\text{ILKAP}}$  from the C2C12 lysates, indicating that ILK-1 and ILKAP are complexed in living cells (Fig 6B). It is important to note that the demonstration of this association does not require overexpression of either binding partner.

We have made ILKAP expression conditional on treatment of cells with the synthetic ecdysone-related hormone, meristerone A. This system was chosen because it provides for very low to absent expression of plasmid-encoded cDNAs in a variety of mammalian cell backgrounds. The H4C11 cDNA was used as a template in a PCR reaction (Pfu polymerase), to generate a 1310 bp product for subcloning into expression vectors. This product contains the 5' untranslated region of H4C11, and removes the stop codon in order to allow fusion of ILKAP sequences to vector-encoded 3' epitope tags, V5 and 6x His. The 1310 bp product was subcloned into the multi-cloning site of pINDtopoV5his (Invitrogen), to produce H4C11-30pINDtopoV5his. Expression of cDNAs from this plasmid is dependent on treatment of the transfectant cells with merA, in the presence of the ecdysone receptor, encoded on the plasmid pVgRXR (Invitrogen). We confirmed that transient co-transfection (Lipofectamine Plus, Life Technologies) of human embryonic kidney 293 (HEK 293) cells with H4C11-30pINDtopoV5his and pVgRXR resulted in

highly inducible expression of V5-tagged ILKAP (Fig. 7A). In order to test the association of ILK-1 and ILKAP in mammalian cells, we performed ILK-1 co-immunoprecipitation assays on the transiently transfected HEK 293 cells. The V5 epitope-tagged p47<sup>ILKAP</sup> band was readily detected in the ILK-1 immune complexes (Fig. 7B). Importantly, although induced levels of tagged ILKAP were high at 6 hr post-induction, significant association with endogenous p59<sup>ILK-1</sup> was not seen until 36 hour post-induction. This demonstrates that the association between ILK-1 and ILKAP is not an artefact of post-lysis binding. We conclude from these data that p59<sup>ILK-1</sup> and p47<sup>ILKAP</sup> are present in stable complexes in the HEK 293 and C2C12 cells, indicating conservation of function across species.

We have developed multiple clones exhibiting conditional expression of p47<sup>ILKAP</sup>. In order to establish independent clones conditionally expressing epitope-tagged ILKAP protein, HEK 293 cells were stably transfected with the pVgRXR vector (Zeocin selectable), which encodes a modified ecdysone receptor. Transfection of H4C11-30pINDtopoV5his into the pVgRXR-293 cells, followed by double-selection in neomycin and zeocin, resulted in multiple independent clones being isolated for characterization of ILKAP expression levels. Twelve clones were analyzed, and found to express variable levels of induced V5-ILKAP protein (Fig. 8). Clones 1, 2, 3, 9 and 10 induce high levels of p47<sup>ILKAP</sup> expression, clones 5, 7 and 12 moderate to low, and clones 4, 6, 8, 11 showed no detectable induction (Fig. 8). Preliminary experiments on the high expressor clones indicates no impairment in adhesion to extracellular matrix proteins, fibronectin and collagen, when p47<sup>ILKAP</sup> is induced. There does appear to be a marked inhibition of these clones in spreading on these matrix substrates, however, suggesting that p47<sup>ILKAP</sup> function may affect aspects of ILK-1 regulation of actin cytoskeletal rearrangements. We are currently testing these clones for effects on adhesion to ECM, spreading, and biochemical markers of ILK signal transduction.

#### B. Development of retroviral vectors expressing ILK-1 variants

Last year's report indicated some progress in the development of retroviral vectors for expressing ILK-1 and related cDNAs in mammalian tissue culture systems. In parallel we have developed the pIND-based constructs for ecdysone-inducible expression in culture, and as we have had much greater success with this approach to expression, have not pursued retroviral delivery further.

## CONCLUSIONS

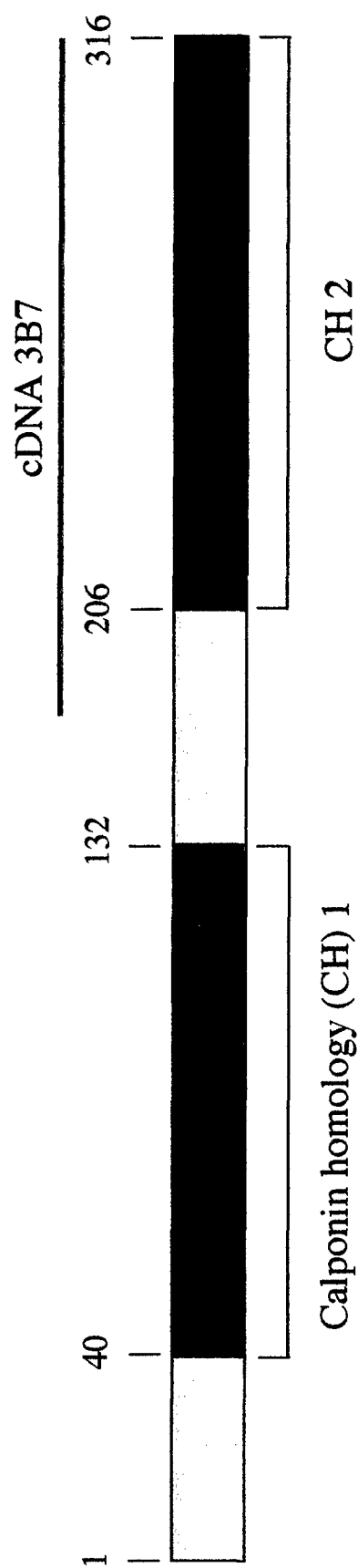
Progress has been made in characterizing two candidate ILK-1 protein partners, using a variety of molecular and biochemical approaches. We have confirmed that p59<sup>ILK-1</sup> and p47<sup>ILKAP</sup> are associated, most likely via direct interaction, in living epithelial cells. This stable complex is likely to be extremely important in determining levels of cellular ILK activity, impinging on downstream effectors such as GSK3- $\beta$  and PKB/AKT. A second novel ILK interactor represents a putative actin binding protein, previously undescribed, which would have important implications for regulation of the actin cytoskeleton by integrins in response to extracellular matrix. This protein may be involved in integrin-induced cell shape changes and/or motility, for example.

ILKAP is likely to function as a protein serine/threonine phosphatase, and we are developing biochemical assays for this function. The association of ILK-1 with a PP2C provides a specific mechanism for the rapid down-regulation of ILK-1-mediated phosphorylation and signalling. Moreover, it provides a novel context for members of the PP2C family, and may suggest other PP2C members are involved in regulating cellular responses to adhesion through non-integrin, or other integrin receptors. Of particular importance, ILKAP likely regulates survival in epithelial cells indirectly, via inhibition of PKB activity. This finding provides us with a novel tool with which to study dysregulation of the ILK-1 signalling pathway in breast carcinoma, but which likely has great relevance to many other carcinomas.

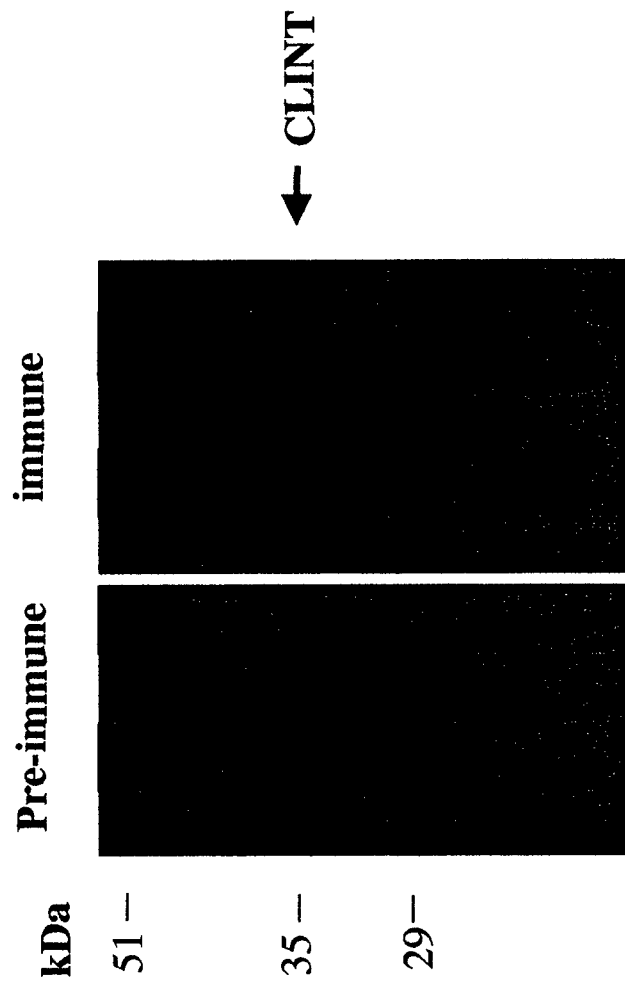
The role of ILK-1 in signalling cell survival appears to involve phosphorylation and activation of PKB, through its PDK2 activity. PKB in turn is thought to be the major cellular protein kinase responsible for phosphorylation and inactivation of BAD, a pro-apoptotic mitochondrial protein. The molecular and biochemical characterization of ILKAP function should allow us to determine whether ILK is the major PDK2 activity in cells of epithelial origin.

**REFERENCES**

1. Hannigan GE, Leung-Hagesteijn C, Fitz-Gibbon L, Coppolino MG, Radeva G, Filmus J, Bell JC, Dedhar S (1996) Regulation of cell adhesion and anchorage-dependent growth by a new  $\beta 1$  integrin-linked protein kinase. *Nature* 379:91-96.
2. Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S (1998) Phosphoinositide-3-OH kinase-dependent regulation of glycogen synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. *Proc Natl Acad Sci USA* 95:11211-11216
3. Dedhar S, Williams B, Hannigan G (1999) Integrin-linked kinase (ILK): a regulator of integrin and growth-factor signalling. *Trends Cell Biol* 9: 319-323.
4. Wang F, Weaver VM, Peterson OW, Larabell CA, Dedhar S, Briand P, Lupu R, Bissell MJ (1998) Reciprocal interactions between beta 1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. *Proc Natl Acad Sci USA* 95:14821-14826.
5. Kusuda K, Kobayashi T, Ikeda S, Ohnishi M, Chida N, Yanagawa Y, Shineha R, Nishihira T, Satomi S, Hiraga A, Tamura S (1998) Mutational analysis of the domain structure of mouse protein phosphatase 2Cbeta. *Biochem J* 332:243-250.
6. Tong Y, Quirion R, Shen SH (1998) Cloning and characterization of a novel mammalian PP2C isozyme. *J. Biol. Chem.* 273:35282-35290.

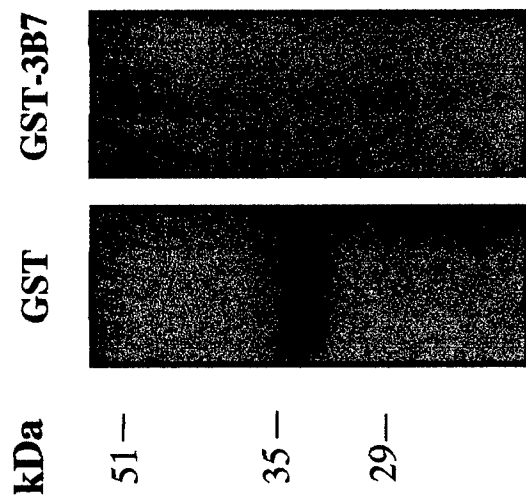


**Figure 1:** Schematic based on the deduced translation product of 3B7 (red line) and est sequences. RT-PCR experiments were performed to amplify mRNA from HEK 293 epithelial cells. Primer pairs representing est sequences just 5' to the putative translation start site (position 1), and 3' 3B7 sequences amplify products of the expected size (ca. 1.7 kb, based on melding est and 3B7 overlapping sequence files), indicating that est and 3B7 sequences reside in the same transcript.

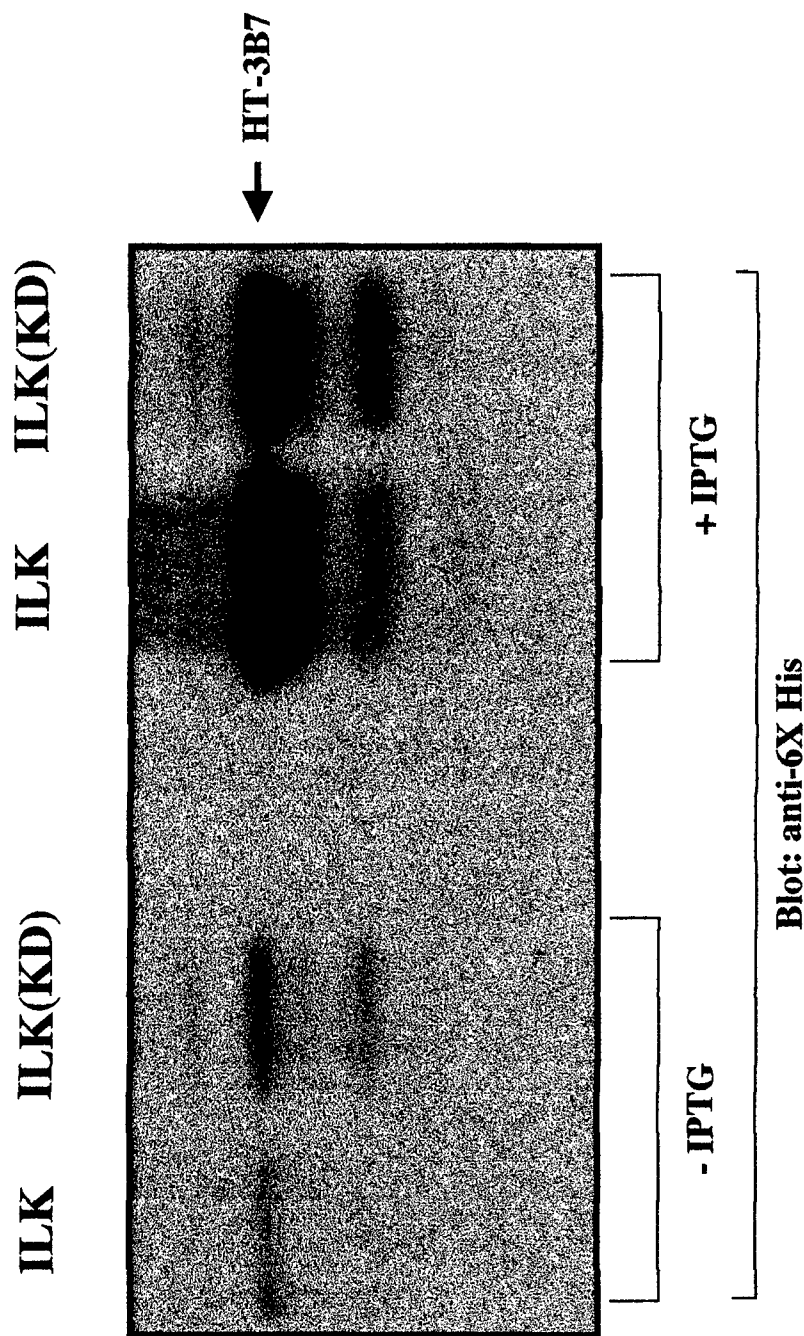


**Figure 2A:** Rabbit polyclonal antiserum, raised against a HT-3B7 fusion protein, was affinity-purified over a column of HT-3B7 fusion protein, covalently coupled to CNBr-activated sepharose. 50  $\mu$ g of HEK 293 cytoplasmic lysate was run on 10% SDS-PAGE, and proteins transferred to PVDF membranes. Equal amounts of HT-3B7 sepharose column eluate from pre- and immune bleeds, were immunoblotted in parallel, and subsequently with a mouse anti-rabbit IgG secondary antibody, conjugated to HRP. Immunoreactive bands were detected using enhanced chemiluminescence (ECL, Amersham). The ca. 35 kDa protein was dubbed CLINT, for Cytoskeletal Linker of INTEgrins.

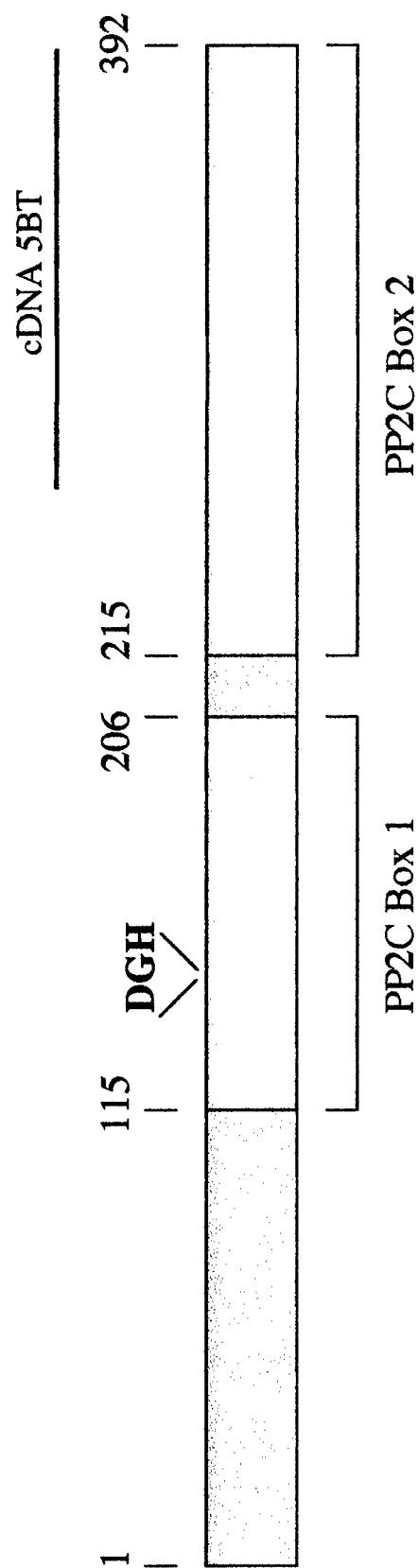




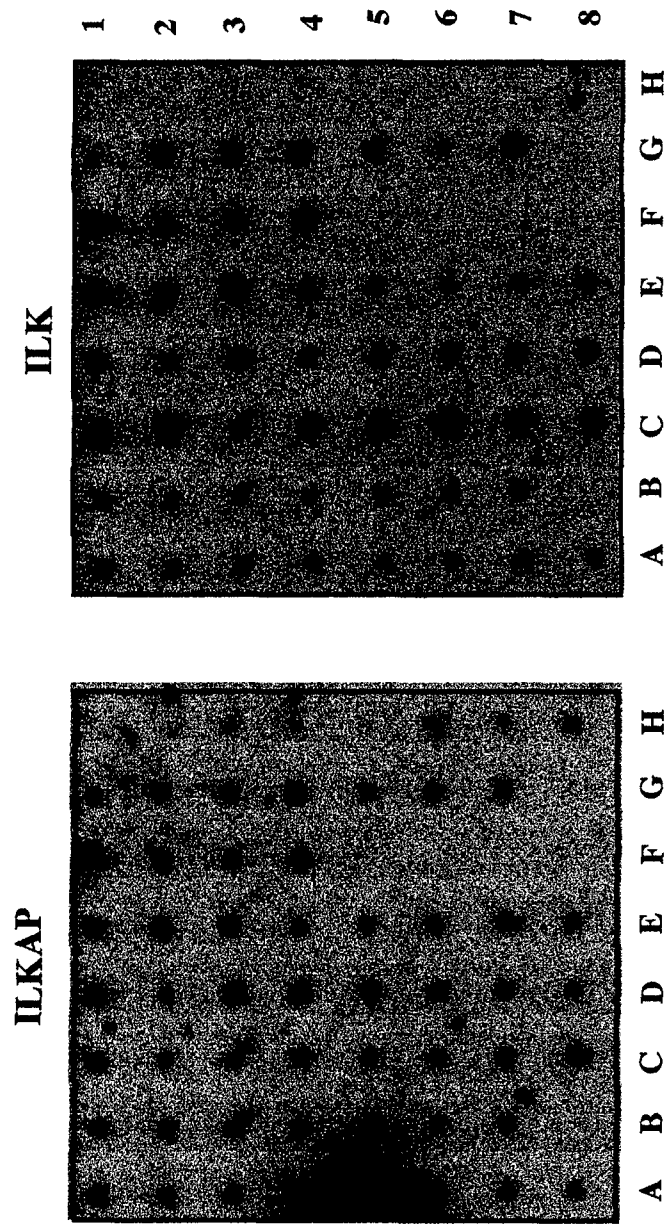
**Figure 2B:** The affinity-purified anti-3B7 antibody (2  $\mu$ g) was incubated with 2  $\mu$ g GST or GST-3B7 recombinant protein. Adsorbed antibodies were used to blot PVDF membranes onto which 50  $\mu$ g of HEK 293 cytoplasmic lysate had been transferred from 10% SDS-PAGE.



**Figure 3:** GST fusion proteins representing full length ILK, and kinase dead ILK (KD), were incubated (1  $\mu$ g) with 30  $\mu$ g cell extract of *E.coli*, BL21(DE3) strain carrying the HT-3B7 expression construct. Lysates from bacteria uninduced (-IPTG) or induced with 1mM IPTG were incubated for 1 hour with the GST-ILK recombinant proteins and then collected on glutathione-agarose beads. Complexes were washed and subjected to 10% SDS-PAGE analysis. Proteins transferred to PVDF membranes were immunoblotted with an anti- 6x His antibody, and visualized as in Figure 2.



**Figure 4:** Schematic of ILKAP translated coding region. A 1.7 kb cDNA was identified from an Origene rapid screen cDNA library. RT-PC was performed, using primers that recognize 5' and 3' flanking cDNA sequences. These experiments confirmed that this transcript is expressed in HEK 293 cells. The red line indicates the peptide encoded by cDNA 5BT. cDNA 17ai is an N-terminally truncated sequence, which does not code for the first 13 amino acid residues of the 5BT-encoded peptide. Our polyclonal antibodies are raised against the C-terminus (17ai-encoded).



**Figure 5.** Clontech Human RNA Master Blot was probed with cDNAs representing full length ILKAP and ILK-1 coding sequences. The normalized amounts of DNA spotted onto each position of the Master Blot provide an indication of the relative levels of expression of ILK-1 and ILKAP mRNA in each of the tissues. Comparison between blots is not applicable because differences in the specific activity of each cDNA probe are not accounted for.

**Key:**

- A. 1: whole brain 2: amygdala 3: caudate nucleus 4: cerebellum 5: cerebral cortex 6: frontal lobe 7: hippocampus 8: medulla oblongata  
 B. 1: occipital lobe 2: putamen 3: substantia nigra 4: temporal lobe 5: thalamus 6: nucleus accumbens 7: spinal cord  
 C. 1: heart 2: aorta 3: skeletal muscle 4: colon 5: bladder 6: uterus 7: prostate 8: stomach  
 D. 1: testis 2: ovary 3: pancreas 4: pituitary 5: adrenal 6: thyroid 7: salivary 8: mammary  
 E. 1: kidney 2: liver 3: small intestine 4: spleen 5: thymus 6: leucocyte 7: lymph node 8: bone marrow  
 F. 1: appendix 2: lung 3: trachea 4: placenta  
 G. 1: fetal brain 2: fetal heart 3: fetal kidney 4: fetal liver 5: fetal spleen 6: fetal thymus 7: fetal lung  
 H. 1: yeast total RNA 2: yeast tRNA 3: E. coli rRNA 4: E. coli DNA 5: poly A+ RNA 6: human Cot 1 DNA  
 7: 100 ng human DNA 8: 500 ng human DNA

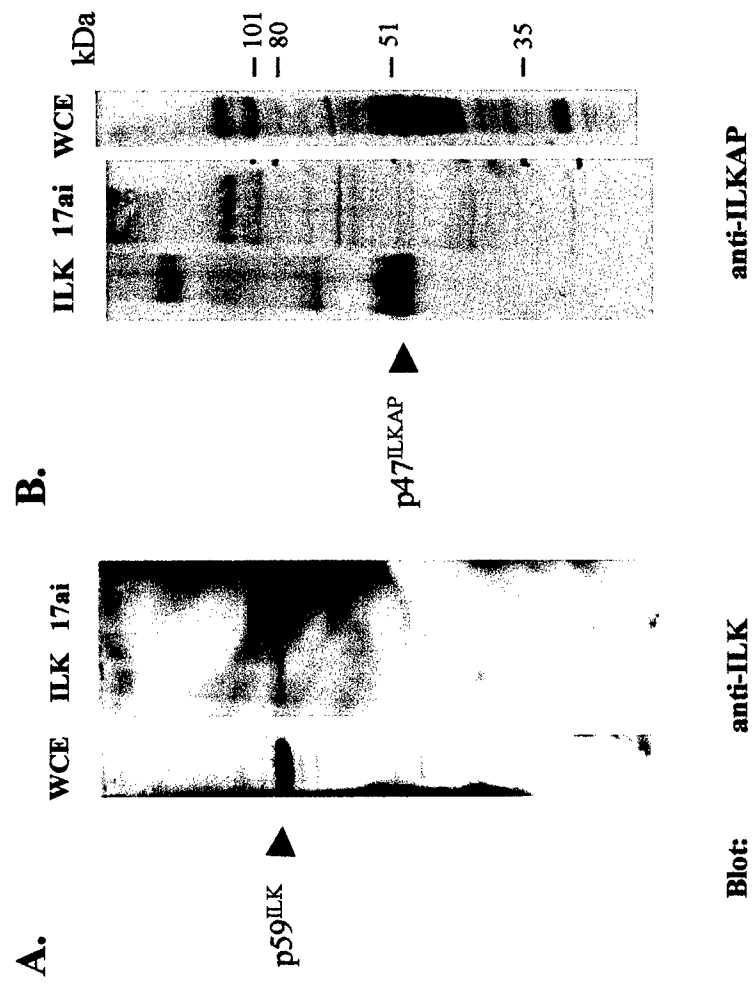
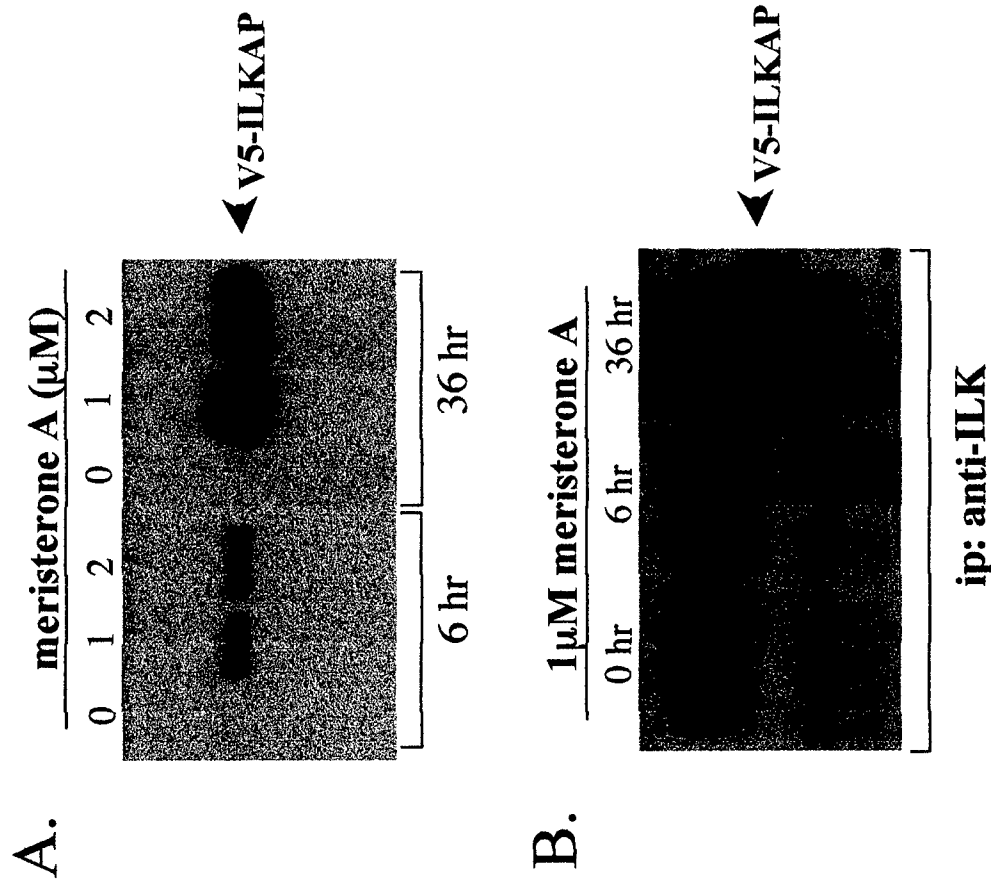
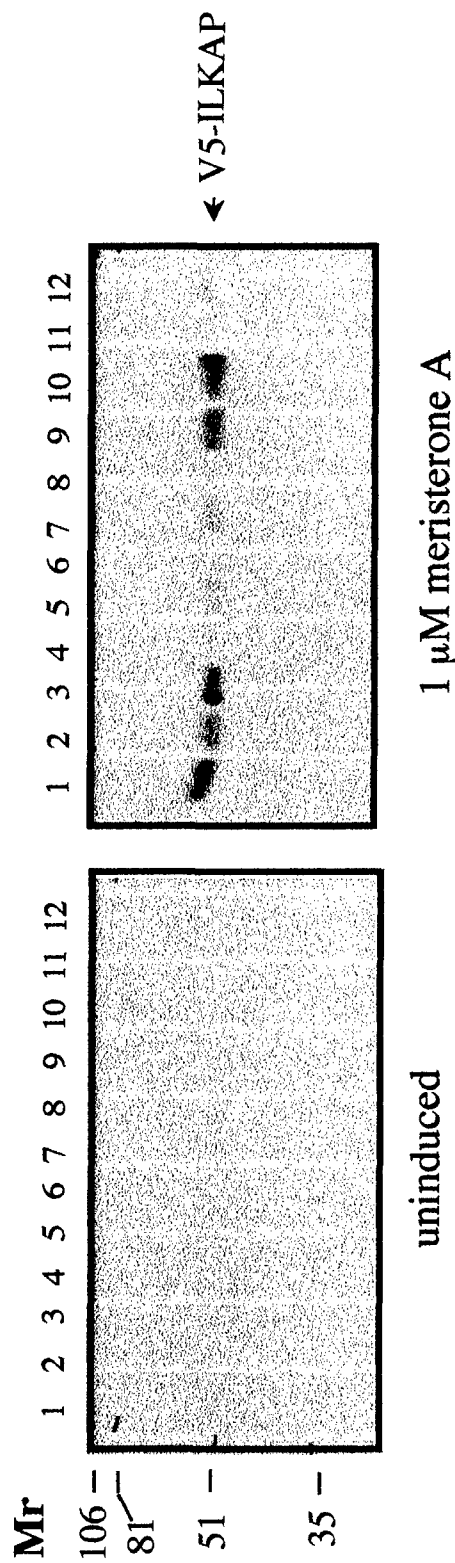


Figure 6.

**Figure 6:** Association of ILK-1 and ILKAP in pull-down and co-immunoprecipitation experiments. Mouse fibroblast C2C12 cytoplasmic lysates (500  $\mu$ g) were immunoprecipitated with 1  $\mu$ g of affinity-purified anti-ILK (ILK lane), or incubated with 1  $\mu$ g of His-tagged 17ai (ILKAP C-terminus, see Figure 4) fusion protein. Immuno-precipitates were collected on Protein A-sepharose and HT-17ai complexes collected on Ni-NTA agarose, washed, and proteins run on 10% SDS-PAGE for transfer to PVDF membranes. The membranes were processed in parallel for western blotting with affinity-purified anti-ILK (A) or affinity-purified anti-ILKAP (B). Whole cell extracts (WCE) were run on the same gels in order to mark migration of cellular ILK and ILKAP proteins.



**Figure 7:** Induction of V5 epitope-tagged ILKAP and association with p59<sup>ILK</sup> in HEK 293 cells. HEK 293 cells were transiently transfected with pINDtopoV5his-ILKAP and pVgRXXR vectors in order to render V5-tagged ILKAP expression meristerone A-dependent. **A.** 30  $\mu$ g total cytoplasmic lysates from the transfectants, treated as indicated, were run on 10% SDS-PAGE, transferred to PVDF and blotted with horseradish peroxidase-conjugated anti-V5. Detection of blots was with ECL. **B.** 100  $\mu$ g of cytoplasmic lysates from transfectant cells treated for 0 hr, 6 hr or 36 hr with 1 mM meristerone A were immunoprecipitated with 1  $\mu$ g affinity-purified anti-ILK, transferred, and blotted with the HRP-conjugated anti-V5 antibody.



**Figure 8:** Stable transfection of inducible ILKAP expression constructs into HEK 293 cells. 293 were co-transfected with the pINDtopo V5his-ILKAP and pVgRXR plasmids. Clones were subsequently grown and double-selected in G418 and Zeocin. Twelve of the isolated stable clones were treated, or not, with meristerone A for 6 hr. 50  $\mu$ g cytoplasmic lysate was run on each lane of a 10% SDS-PAGE gel, and transferred to PVDF membranes. Detection of the tagged ILKAP was through use of the HRP-conjugated anti-V5 antibody in standard western blots.





**DEPARTMENT OF THE ARMY**  
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND  
504 SCOTT STREET  
FORT DETRICK, MARYLAND 21702-5012

REPLY TO  
ATTENTION OF:

MCMR-RMI-S (70-1y)

27 Sep 02

MEMORANDUM FOR Administrator, Defense Technical Information  
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,  
VA 22060-6218


SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for Accession Document Numbers ADB264654, ADB241161, and ADB253101 be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

  
PHYLLIS M. RINEHART  
Deputy Chief of Staff for  
Information Management